EXHIBIT 2

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Glucose ENFET doped with MnO_2 powder

Li-Te Yin[a], Jung-Chuan Chou[b,*], Wen-Yaw Chung[c], Tai-Ping Sun[d], Kuang-Pin Hsiung[e], Shen-Kan Hsiung[c]

[a]*Department of Bio-Medical Engineering, Chung Yuan Christian University, Chung-Li, Taiwan 320, ROC*
[b]*Institute of Electronic and Information Engineering, National Yunlin University of Science and Technology, Touliu, Taiwan 640, ROC*
[c]*Department of Electronic Engineering, Chung Yuan Christian University, Chung-Li, Taiwan 320, ROC*
[d]*Department of Electrical Engineering, National Chi Nan University, Nantou, Taiwan 545, ROC*
[e]*Union Chemical Laboratories, Industrial Technology Research Institute, Hsinchu, Taiwan 300, ROC*

Abstract

In this study, a glucose-sensitive enzyme field-effect transistor (ENFET) coimmobilized with glucose oxidase (GOD) and manganese dioxide (MnO_2) have been investigated. The biomembrane of the ENFET was immobilized on the amorphous tin oxide/indium tin oxide glass structure extended sensitive gate, which used as a disposable transducer of a glucose biosensor. MnO_2 was used as a catalyst which can catalyze the hydrogen peroxide and produced H_2O and O_2. Coimmobilizaion of glucose oxidase and manganese dioxide was found to be useful for extending the dynamic measured range of glucose concentration to 360 mg/dl (eq. 20 mM). The result shows that the dynamic range of the output signal is strongly dependent on pH value of measuring environments, and the measurement in the alkali buffer solutions shows a higher response and wider dynamic range. Additionally, the different immobilized layers of MnO_2 have been studied. The MnO_2, which be immobilized in outer cross-linking layer of bovine serum albumin, shows better results than immobilized in GOD layer or gultaraldehyde covalent layer. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Extended gate field effect transistor (EGFET); Enzyme field effect transistor (ENFET); Amorphous tin oxide; Glucose oxidase (GOD); Manganese dioxide (MnO_2)

1. Introduction

Since the first reported enzyme biosensor (ENFET) based on ion-sensitive field effect transistors (ISFETs) [1], substantial research efforts were undertaken to improve the performance characteristics of the ENFET's developed. Until now, there are almost two dozen papers dealing with glucose ENFETs which suffer from many problems [2].

Normally glucose oxidase hydrolyzes glucose according to the following reactions:

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\beta\text{-D-glucose oxidase}} \text{D-glucono-}\delta\text{-lactone} + H_2O_2 \quad (1)$$

$$\text{D-glucono-}\delta\text{-lactone} \rightarrow \text{D-gluconate} + H^+ \quad (2)$$

ISFET sensors measure the glucose concentration by detecting the pH variation due to the hydrogen ions that are generated by the dissociation of gluconic acid. However, because of the low dissociation constant ($pK_a \cong 3.8$) [3], ISFET glucose sensors show low sensitivities. Generally, the sensitivities at the physiological pH value are limited to only some millivolts per decade [4]. Hence the ISFET drift, which is an inherent characteristic of ISFETs, becomes a important topic. The glucose concentration in human blood is normally about 5 mM, reaching 20 mM and more for diabetics. However, the concentration of oxygen, does not exceed 0.5 mM. Because of the unfavourable concentration ratio of glucose and oxygen in real blood, the dynamic range of the biosensor is usually limited by oxygen and dose not exceed several mM. Since, the oxygen in the sensor membrane is consumed by the enzyme reaction, the oxygen concentration is needed high enough for a better linearity between output voltage and the glucose concentration. Moreover, the hydrogen peroxide, one of the by-products of the glucose oxidation, acts an inhibitor of glucose oxidase which causes the lower sensitivity and bad repeatability in the steady measurement system of glucose ENFET.

Sudoh et al. employed pre-electrolysis method to enrich the oxygen of the glucose solution, which the oxygen is generated by electrolysis of the solution before monitoring

* Corresponding author. Tel.: +886-553-42601/ext. 2500; fax: +886-553-12029.
E-mail address: choujc@pine.yuntech.edu.tw (J.-C. Chou).

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[5]. The output signal was linearly proportional to the glucose concentration up to 500 mg/dl by the supplement of oxygen generated by electrolysis of the solution. Seo et al. and Lee et al. employed a Pt electrode actuator on the ISFET sensitive gate to electrolyze the hydrogen peroxide [2,6]. The sensor with the Pt electrode actuator exhibits a wide dynamic range that from 1 to 10 mM. Saito et al. used an external BSA membrane, which is highly cross-linked by glutaraldehyde, to restrict glucose diffusion to expand the measuring range and make a stable response in a low buffer capacity solution [7]. The sensor outputs shows a good linear relationship with up to 300 mg/dl glucose concentration. Shul'ga et al. added the potassium ferricyanide, which used as an oxidizing substrate in the biocatalytic oxidation of glucose, into the phosphate and TRIS buffer to perform the glucose ENFET measurement [8]. Depending on the concentration of ferricyanide the glucose ENFET shows a 10–100 times increase of the biosensor response and a substantial extension of its dynamic range.

In this paper a glucose sensor based on H^+-ion-sensitive field effect transistor (ISFET) has been realized in combination with a MnO_2-doped glucose oxidase membrane. Zheng and Guo brought forward the following procedures and reactions of H_2O_2 catalyzed by MnO_2 [9]:

$$MnO_2 + H_2O \rightarrow MnO(OH)_2 \quad (3)$$

$$MnO(OH)_2 \rightarrow Mn^{2+} + 2OH^- \quad (4)$$

$$Mn^{2+} + H_2O_2 + 2OH^- \rightarrow MnO_4^{2-} + H_2O \quad (5)$$

$$MnO_4^{2-} \rightarrow MnO_2 + O_x \quad (6)$$

Where MnO_2 was used as a catalyst which can catalyze the hydrogen peroxide and produce H_2O and O_2. In addition to the reduction of H_2O_2 concentration in the biolayer, the product, oxygen, can be recycled for glucose oxidation reaction. The MnO_2 doping position and the pH value of working have been investigated.

2. Experimental

2.1. Chemicals and materials

The β-D-glucose oxidase (GOD) EC 1.1.3.4 from *Aspergillus niger*, bovine serum albumin from Serva and γ-aminopropyl triethoxysilane (3-APTS, 99%) were purchased from Sigma. Glutaraldehyde (GA, 25% aqueous solution) was purchased from Acros Organics. Manganese dioxide powder (99.9%) was obtained from Tekstart (Hsinchu, Taiwan). All other regents were in reagent grade and were used without further purification. Distilled water was used for all the electrolytes and the buffer solutions. Tin oxide thin films were formed by the RF sputtering system (tin oxide target, 99.9%) at a substrate temperature of 150°C. The ITO glasses (50–100 Ω/sq; ITO coating thickness, 230 Å) were supplied by the Wintek Corporation.

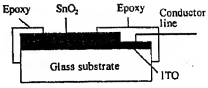

Fig. 1. Cross-section of SnO_2/ITO glass sensing structure.

2.2. Sensor Fabrication

2.2.1. Solid-state part

In this study, the glucose ENFET is based on a separative extended gate ISFET (EGFET) structure. The sensitive part of the separative EGFET is shown as Fig. 1. The SnO_2 thin film was deposited by using sputtering method with a thickness of 2000 Å. Before the glass was deposited SnO_2, it was washed in methyl alcohol and DI water for 20 and 10 min, respectively. The SnO_2/ITO glass EGFET shows a linear pH response about 57 mV/pH between pH 2.4 and pH 11.2 [10].

2.2.2. Enzyme immobilization

The procedure for preparation of separative structure of ENFET is as follows.

1. Cleaning: The separative structure of EGFET was cleaned by distilled water.
2. Silanization: There is no amino group on our sensitive film, so we use 3-APTS to modify tin oxide (SnO_2) substrate. The procedure is represented as follow [11,12]:

$$SnO_2 \xrightarrow{\text{silanizaton}} SnO_x{-}Si(CH_2)_3NH_2$$

3. Activation by glutaraldehyde: Glutaraldehyde (1%) is also used extensively to immobilize enzyme molecules onto a carrier substance bearing amino group. The procedure is represented as follow:

$$SnO_x{-}Si(CH_2)_3NH_2 \xrightarrow{\text{glutaraldehyde}} SnO_x{-}Si(CH_2)_3 N{=}CH{-}(CH_2)_3{-}CHO$$

4. Coupling of the enzyme and cross-linking: The GOD (40 mg) was dissolved in 1 ml of a 0.1 M K–P buffer solution (pH 7.0). A 1.5 μl part of the solution was cast onto the gate region and then addition of 1 μl of the glutaraldehyde was followed to chemically cross-link the membrane. The procedure is represented as follow:

$$SnO_x{-}Si(CH_2)_3N{=}CH{-}(CH_2)_3{-}CHO \xrightarrow{\text{enzyme}} SnO_x{-}Si(CH_2)_3N{=}CH{-}(CH_2)_3{-}CH{=}N{-}(\text{enzyme})$$

5. The outer BSA membrane doping with MnO_2: An amount of 10 mg MnO_2 was dissolved in 300 mg/dl BSA and 6% glutaraldehyde (1:1) solution. A 1 μl part of the solution was cast onto the enzyme membrane.

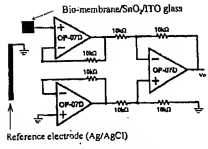

Fig. 2. Separative extended ENFET measurement circuit with instrumentation amplifier LT1167.

In the study of the effect of MnO_2 doping position, the outer BSA membrane was not immobilized, and the MnO_2 was doped in activation or GOD with 10 mg/ml MnO_2.

2.3. Measurement

A readout circuit based on an instrumentation amplifier LT1167 is shown in Fig. 2. The measurement configuration consists of a separative extended gate with biomembrane and the Ag/AgCl reference electrode. The instrumentation amplifier, LT1167, was a transducer and the small output voltage will depend on the pH value. HP3478A and HP VEE program were designed and used as an Y–T recorder to record the voltage variation with time. All measuring temperature of our experiments are in 25°C, 5 mM phosphate–KOH buffer.

3. Results and discussion

3.1. Glucose ENFET response

In this study, a separative sensitive gate of biomembrane/SnO_2/ITO glass structure was used as a disposable biochemical transducer. This structure has advantages of light insensitivity, easier fabrication processes than traditional ISFET and lower cost than SOS structure ISFETs or silicon based EGFET [10]. Fig. 3 shows the pH response of separative sensitive structure with biomembrane of sensitivity 58.3 mV/pH between pH 2 and 10.

Figs. 4 and 5 show typical time response curve for the glucose ENFET without and with the outer BSA membrane. The glucose ENFET was immersed in blank buffer solution for 1min and then immersed in glucose solution. As the ENFET are measured in blank buffer, it shows a drift that not exceeds to 1 mV for 1 min. The glucose ENFETs without and with BSA membrane show response time of 5 and 12 min, respectively. The glucose ENFET, which has outer MnO_2-doped BSA membrane, shows a good linearity up to 360 mg/dl, because the O_2 concentration in biomembrane was greatly rose by MnO_2 catalyzing H_2O_2. The relationship between the sensor output and glucose concentration is shown in Fig. 6.

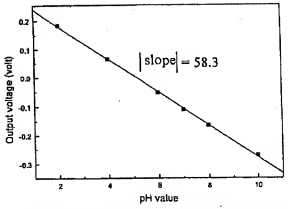

Fig. 3. Output voltage vs. pH value for the biomembrane/SnO_2/ITO glass sensing gate connected with instrumentation amplifier LT1167.

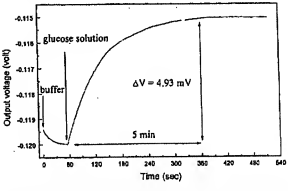

Fig. 4. Response of the separative structure of the ENFET without outer BSA membrane to detect 40 mg/dl glucose in pH 7.2 buffer solution. MnO_2 was immobilized in GOD layer.

Saito et al. employed the glucose ENFET with external BSA membrane, which is very similar our device, shows a good linear relationship with up to 300 mg/dl glucose concentration [7] which the experiments are performed in a

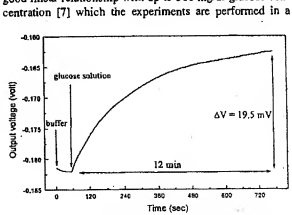

Fig. 5. Response of the separative structure of the ENFET with outer BSA membrane to detect 45 mg/dl glucose in pH 8.5 buffer solution. MnO_2 was immobilized in BSA layer.

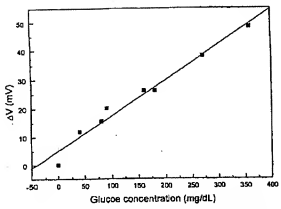

Fig. 6. Calibration curve for the glucose ENFET with outer MnO_2-doped BSA membrane. Sensors were measured in pH 8.1, 5 mM buffer solution.

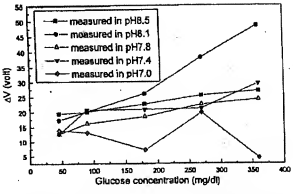

Fig. 8. Effect of pH on the glucose ENFET with outer MnO_2-doped BSA membrane. The curves correspond to different pH values of measured environments: (■) pH 8.5; (●) pH 8.1; (▲) pH 7.8; (▼) pH 7.4; (◆) pH 7.0.

stirring status. But, all the measurements in this paper are performed in a steady status. Fig. 7. shows the sensor response for sensors with non-doped MnO_2 outer BSA layer and MnO_2-doped BSA layer. The results show that sensors with MnO_2-doped BSA show wider dynamic range than sensors with non-doped MnO_2 outer BSA layer. The sensors of non-MnO_2-doped show a high response in lower glucose concentration, but very low response in high glucose concentration.

3.2. Effect of pH on the ENFET response

According to the report of Zheng and Guo, in the experiment of potentiometric determination of hydrogen peroxide at MnO_2-doped carbon paste electrode, while the pH changed in the range 7.0–8.0, the potential response increased with increasing pH [9]. The results may be related to the enhancing of oxidizing ability of H_2O_2 when pH changed in this range. For pH values in the range of 8.0–9.0, the response was almost constant. In our research, the effect of pH on the glucose ENFET response shows the best results of sensitivity and linearity that are shown in Fig. 8. As the GOD catalyzes glucose, the actual pH value of the ENFET biomembrane is lower than the pH value of buffer. The output signal of the measurement in pH 8.5 is lower than that of in pH 8.1 which is caused by that the activity of the GOD is bad in alkali [13,14]. In addition, the results measured in lower pH environments show a bad linearity which is caused by that the MnO_2 shows lower catalysis ability in acid.

3.3. Effect of MnO_2 doping position

As mention before, while the pH changed in the range 7.0–8.0, the catalysis ability of MnO_2 increased with increasing pH value. However, the actual pH value is different in individual biomembrane layer as the ENFET dips into glucose solution. The GOD layer shows the lowest pH that caused by the glucose catalyzed and producing H^+-ion. The H^+ will diffuse into the activation layer and outer layer, which close to the SnO_2 sensitive film and pH-buffer

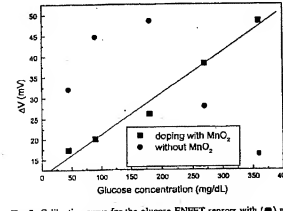

Fig. 7. Calibration curve for the glucose ENFET sensors with (●) non-doped MnO_2 outer BSA layer and (■) MnO_2-doped BSA layer. Sensors were measured in pH 8.1, 5 mM buffer solution.

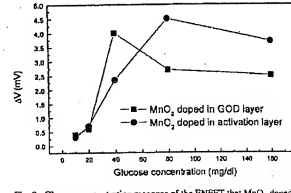

Fig. 9. Glucose concentration response of the ENFET that MnO_2-doped in the GOD layer and in the activation layer, respectively.

solution, respectively. By the effect of the carrier-mediated transport of protons, the outer membrane will show the highest pH value, which is better suitable condition for the reaction between MnO_2 and H_2O_2. As the results shown above, the glucose ENFET that biomembrane with outer MnO_2-doped BSA membrane has a linear dynamic range to 360 mg/dl. Fig. 9, shows the glucose response of MnO_2-doped in the activation layer and the GOD layer. The responses are limited in the high glucose concentration, which caused by that the reaction between MnO_2 and H_2O_2 was blocked in acid environments, especially the response of the device was MnO_2-doped in enzyme layer.

4. Conclusions

A glucose ENFET based on a new principle, in which the biomembrane was doped with MnO_2 powder. MnO_2 was used to perform the catalysis of hydrogen peroxide (one of the by-products of glucose oxidation), was proposed and its characteristics were investigated. The sensor shows a wide dynamic range to the glucose concentration of 360 mg/dl. Both the pH value of buffer solution and MnO_2-doped position affect the response of the glucose ENFET. For the MnO_2-doped effect, both the responses of MnO_2-doped in the activation layer and the GOD layer are limited in the high glucose concentration, which is caused by that the reaction between MnO_2 and H_2O_2 blocked in acid environments, especially the response of the device that MnO_2-doped in the enzyme layer. For the effect of pH value of buffer solution, the glucose ENFET, which MnO_2-doped in the outer BSA layer, measured in pH 8.1 has the largest response and the widest dynamic range in our experiments. In addition, the sensors with MnO_2-doped BSA show wider dynamic range than sensors with non-doped MnO_2 outer BSA layer.

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Biographies

Li-Te Yin was born in Taipei, Taiwan, Republic of China, on December 5, 1973. He received bachelor degree in electrical engineering and master degree in aeronautical and astronautical engineering from Chung-Hua Polytechnic Institute, Hsinchu, Taiwan, in 1995 and 1997, respectively. Since 1997, he has been working toward the PhD degree in the Department of BioMedical Engineering at Chung Yuan Christian University, Chung-Li, Taiwan. His research interests are in the biosensors.

Jung-Chuan Chou was born in Tainan, Taiwan, Republic of China, on July 13, 1954. He received his BS degree in physics from Kaohsiung Normal College, Kaohsiung, Taiwan, in 1976; the MS degree in applied physics from Chung Yuan Christian University, Chung-Li, Taiwan, in 1979; and the PhD degree in electronics from National Chiao Tung University, Hsinchu, Taiwan, in 1988. He taught at Chung Yuan Christian University from 1979 to 1991. Since 1991, he has worked as an associate professor in the Department of Electronic Engineering at the National Youlin University of Science and Technology. His research interests are in the areas of amorphous materials and devices, electrographic photoreceptor materials and devices, electronic materials and devices, sensor devices, and science education.

Wen-Yaw Chung was born in Hsin-Chu, Taiwan, ROC, on March 15, 1957. He received the BSEE and MS degrees from Chung Yuan Christian University, Chung Li, Taiwan, in 1979 and 1981, respectively, and the PhD degree in Electrical and Computer Engineering from Mississippi State University, USA, in 1989. Subsequently, he joined the Advanced Microelectronics Division, Institute for Technology Development in Mississippi, where he was involved in the design of a bipolar optical

data receiver. In 1990, he worked as a design manager for the Communication Product Division, United Microelectronics Corporation, Hsin-Chu, where he was involved in the design of analog CMOS data communication integrated circuits. Since 1991, he has been an associate professor in the Department of Electronic Engineering of Chung Yuan Christian University. His research interests include mixed-signal VLSI design, biomedical IC applications, and test-chip design methodology for deep submicron VLSI electronics.

Tai-Ping Sun was born in Taiwan on March 20, 1950. He received the BS degree in electrical engineering from Chung Cheng Institute of Technology, Taiwan, in 1974, the MS degree in material science engineering from National Tsing Hua University, Taiwan, in 1977, and PhD degree in electrical engineering from National Taiwan University, Taiwan, in 1990. From 1977 to 1997, he worked at Institute of Science and Technology, Republic of China, concerning the development of infrared device, circuit and system. He joined the Department of Management Information System, Chung-Yu College of Business Administration since 1997 as a associated professor. Since 1999, he has joined the Department of Electrical Engineering, National Chi Nan University as a professor and his research interests have been in infrared detector and system, analog/digital mixed-mode integrated circuit design, special semiconductor sensor and their application.

Kuang-Pin Hsiung was born on August 12, 1944. He received the BS degree from Department of Chemistry, Tamkang Arts and Sciences College in 1967, and the PhD degree from Department of Chemistry, Louisiana State University, USA, in 1974. From 1977 to 1985, he was a researcher in Department of Agriculture Products Utilization, Union Industrial Laboratories, Industrial Technology Research Institute. Since 1985, he has been a senior researcher in Union Chemical Laboratories, ITRI. His current research interests are acute myocardial infarction, AMI, serum markers diagnostic kits, point-of-care-test immunoassay methodology & instrumentation, dedicate function clinical analyzers, polyunsaturated fatty acid derivatives and direct electron transfer biological reactions.

Shen-Kan Hsiung was born on June 14, 1942. He received the BS degree from Department of Electrical Engineering, National Cheng-Kung University, in 1965, the MS degree from Department of Electronic Engineering, National Chiao-Tung University, Taiwan, in 1968 and the PhD degree from Material Science Engineering of USC, USA, in 1974. From 1974 to 1978, he was an associate professor in Department of Electrical Engineering, Chung Yuan Christian University. Since 1978, he has been a professor in Department of Electronic Engineering, Chung Yuan Christian University. His current interests are electronic materials, amorphous thin films and semiconductor sensors.